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## HIGH BLOOD-PRESSURE IN TRANSGENIC MICE CARRYING THE RAT ANGIOTENSINOGEN GENE

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# High blood pressure in transgenic mice carrying the rat angiotensinogen gene

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**Transgenic mice were generated by injecting the entire rat angiotensinogen gene into the germline of NMRI mice. The resulting transgenic animals were characterized with respect to hemodynamics, parameters of the renin angiotension system, and expression of the transgene. The transgenic line TGM(rAOGEN)123 developed hypertension with a mean arterial blood pressure of 158 mmHg in males and 132 mmHg in females. In contrast, the transgenic line TGM(rAOGEN)92 was not hypertensive. Rat angiotensinogen was detectable only in plasma of animals of line 123. Total plasma angiotensinogen and plasma angiotensin II concentrations were about three times as high as those of negative control mice. In TGM(rAOGEN)123 the transgene was highly expressed in liver and brain. Transcripts were also detected in heart, kidney and testis. In TGM(rAOGEN)92 the brain was the main expressing organ. *In situ* hybridization revealed an mRNA distribution in the brain of TGM(rAOGEN)123 similar to the one in rat. In TGM(rAOGEN)92 the expression pattern in the brain was aberrant. These data indicate that overexpression of the angiotensinogen gene in liver and brain leads to the development of hypertension in transgenic mice. The TGM(rAOGEN)123 constitutes a high angiotensin II type of hypertension and may provide a new experimental animal model to study the kinetics and function of the renin angiotensin system.**

**Key words:** angiotensinogen/brain/hypertension/liver/transgenic mice

## Introduction

The renin angiotensin system (RAS) is the most important regulator of blood pressure and body electrolyte homeostasis. Angiotensinogen (AOGEN), the only high molecular weight protein precursor of angiotensins, is cleaved by renin to angiotensin I (ANG I), which is subsequently converted by the action of converting enzyme to the potent vasoconstrictor octapeptide hormone angiotensin II (ANG II) (for reviews see Peach, 1977; Dzau and Pratt, 1986; Lindpaintner and Ganten, 1991). The main source of plasma AOGEN is the liver (Campbell *et al.*, 1984), where its synthesis and release

into the circulation is regulated in response to a number of different stimuli such as steroid hormones (Chang and Perlman, 1987; Feldmer *et al.*, 1991), cytokines (Brasier *et al.*, 1990; Ron *et al.*, 1990a) and ANG II (Klett *et al.*, 1988a,b). In the past decade the existence of local tissue RAS has been established. Gene expression of components of the RAS, including AOGEN has been demonstrated in a large number of extrahepatic tissues, among them the brain, heart and kidney (Ohkubo *et al.*, 1986; Deschepper *et al.*, 1986; Campbell and Habener, 1987; Dzau *et al.*, 1987; Hellmann *et al.*, 1988; Stornetta *et al.*, 1988). The transcription of the AOGEN gene in these tissues seems to be regulated in a cell type specific manner (Campbell and Habener, 1986). *In situ* hybridization of rat brain revealed a predominant expression of the AOGEN gene within nuclei related to cardiovascular control (Lynch *et al.*, 1986; Bunnemann *et al.*, 1990). However, the functional relevance of locally generated ANG II in the development of hypertension is not yet fully understood.

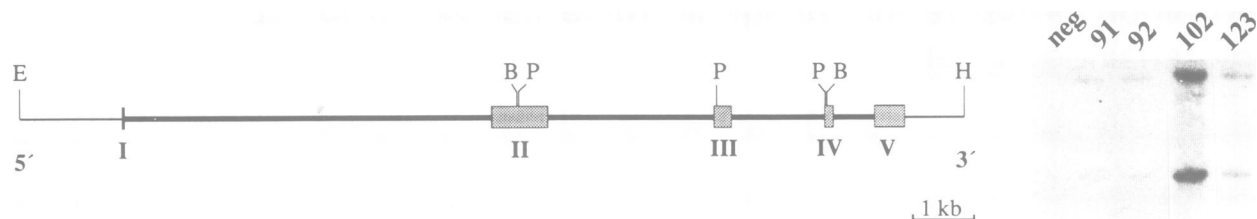
An increasing number of studies has successfully employed transgenic animal models to elucidate the participation of candidate genes in the development of pathophysiological changes within the organism (Hsiao *et al.*, 1990; Hammer *et al.*, 1990; Ryan *et al.*, 1990; Weiher *et al.*, 1990). The generation of hypertensive transgenic rats harbouring the mouse renin gene (Mullins *et al.*, 1990) for the first time provided evidence for a local paracrine mechanism in the development of hypertension. These rats have extremely high blood pressure with low circulating renin and ANG II levels.

To investigate whether the overexpression of the AOGEN gene can also lead to the development of high blood pressure, we generated transgenic mice carrying the rat AOGEN gene. The mouse is particularly suited for these experiments because it is known to have low circulating AOGEN concentrations and because the mouse renin is capable of cleaving rat AOGEN. The use of a heterologous gene (mouse versus rat) allows the differential analysis of the endogenous versus the transgene. We obtained different lines of transgenic mice and report here on the characteristics of these animals with respect to plasma parameters, hemodynamics and the expression pattern of the transgene. We demonstrate that the transgenic mouse line TGM(rAOGEN)123 expresses high levels of the transgene in liver and brain, has elevated plasma AOGEN and ANG II concentrations, and develops hypertension.

## Results

### Generation of transgenic mice

The genomic rat AOGEN clone pRag15U (Tanaka *et al.*, 1984) (Figure 1), including 1.6 kb of 5' flanking sequences, was microinjected into fertilized eggs of NMRI mice as a 14.2 kb linear *EcoRI*–*HindIII* fragment. Three foster mothers carried 16 mice to term, four of which carried the transgene as analysed by Southern blotting of DNA obtained



**Fig. 1.** Southern blot analysis of transgenic mice. Schematic map of the 14.2 kb *EcoRI* (E)–*HindIII* (H) fragment of pRag15U used for microinjection of NMRI mice. Hatched boxes, designated I–V, represent the five exons of the rat AOPEN gene, which are interrupted by four introns (thick solid lines). Thin lines represent 1.6 kb of 5' and 0.9 kb of 3' flanking sequences, respectively. *Bam*HI (B) and *Pvu*II (P) restriction sites, which are relevant for Southern analysis, are indicated. Genomic mouse DNA was digested with *Pvu*II and hybridized to a 712 bp *Bam*HI fragment of the rat AOPEN cDNA. The appearance of two hybridization signals is indicative of the presence of the transgene. Numbers above the lanes indicate the transgenic lines that were established from the particular founder animal. The left lane (neg) contained DNA of a transgene negative litter-mate.

from tail biopsies (Figure 1). Three of these founder animals transmitted the transgene to their progeny and the transgenic lines, TGM(rAOPEN)92, 102 and 123 were established.

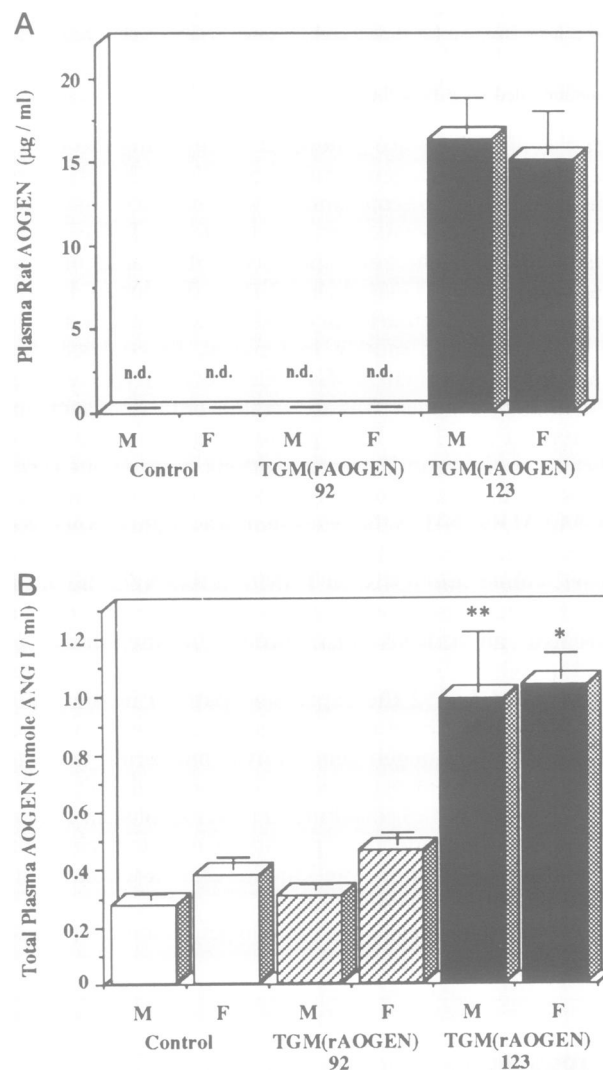
RNAse protection analyses of total liver RNA of animals of these three lines showed that the transgene was highly expressed in TGM(rAOPEN)102 and 123 (Figure 5 and data not shown). We chose TGM(rAOPEN)123 for further investigation, because the transgene segregated in a Mendelian manner as opposed to TGM(rAOPEN)102, in which there seemed to be multiple insertion sites obviously on different chromosomes. This resulted in a complex genetics in the F2 generation. Expression of the transgene in the liver of animals of line 92 was very weak (Figure 5). All experiments reported here were carried out with heterozygous animals of transgenic lines 92 and 123.

#### **Transgenic mice have elevated AOPEN and ANG II levels**

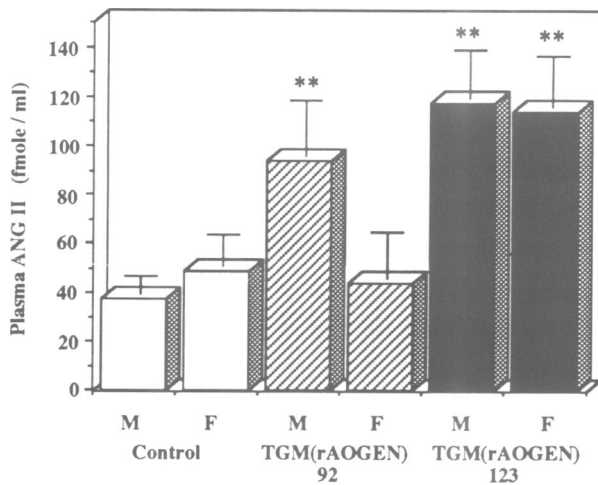
To test whether the expression of the rat AOPEN gene in the liver of transgenic mice results in elevated circulating protein concentrations, we measured several plasma parameters of the RAS. Using the ELISA technique with the rat AOPEN specific monoclonal antibody A1-F6 we detected high levels of rat AOPEN in the plasma of TGM(rAOPEN)123 with no significant difference between females ( $15.1 \pm 2.55 \mu\text{g/ml}$ ) and males ( $16.3 \pm 2.14 \mu\text{g/ml}$ ) (Figure 2A). However, as in negative control animals, no specific reaction of the antibody with plasma proteins of TGM(rAOPEN)92 could be detected (Figure 2A). This indicates that the concentration of rat AOPEN in the plasma of TGM(rAOPEN)92 is below the limit of detection of the ELISA and that the antibody does not cross-react with the mouse AOPEN.

The total rat and mouse plasma AOPEN concentration was measured by indirect RIA via the generation of ANG I. Animals of TGM(rAOPEN)123 showed >2-fold higher total substrate concentrations than those of TGM(rAOPEN)92 or transgene negative litter-mates (Figure 2B). Again there was no difference between male and female animals. Since the data for rat and total AOPEN concentrations were obtained with different (direct and indirect) methods, it is not possible to calculate the exact contribution of rat AOPEN to the concentration of total AOPEN.

Plasma ANG II concentrations were determined by direct radioimmunoassay (RIA) and found to be about three times higher in TGM(rAOPEN)123 than in normal mice (Figure



**Fig. 2.** AOPEN concentration in plasma of transgenic mice. (A) Plasma rat AOPEN concentrations were determined by ELISA using the monoclonal antibody A1-F6 (see Materials and Methods). (B) Total AOPEN concentrations were measured by indirect RIA via the generation of ANG I. Plasma aliquots were incubated for 3 h with a kidney homogenate of NMRI mice. Columns represent the mean  $\pm$  SEM of values obtained from measurements of plasma samples of six or more male (M) or female (F) animals, each carried out in two parallel determinations. n.d., not detectable, asterisks indicate significant differences to the respective negative control as stated in Materials and Methods.



**Fig. 3.** ANG II concentration in plasma of transgenic mice. Plasma ANG II concentrations were determined by direct RIA. Calculation and presentation of data is as in Figure 2.

3). Interestingly, male but not female animals of TGM(rAOGEN)92 also had elevated plasma ANG II concentrations (Figure 3).

#### **Transgenic mice develop high blood pressure**

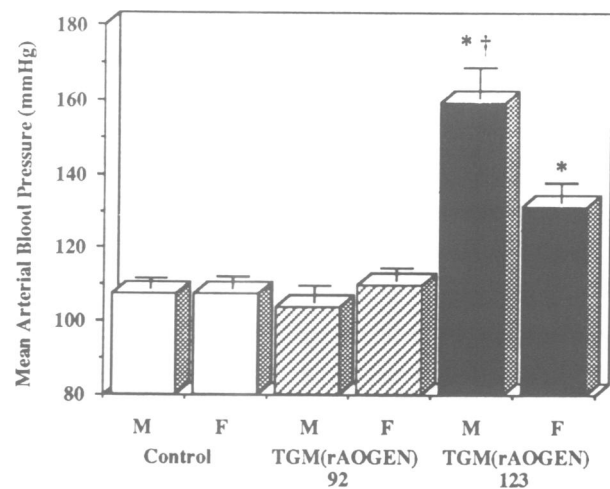
Since animals of TGM(rAOGEN)123 had increased circulating substrate and ANG II level, we measured the mean arterial blood pressure (MAP) to determine whether the increased effector peptide concentrations have hemodynamic effects. The data in Figure 4 were obtained by measuring the MAP in five or more different animals of each group. Clearly, animals of TGM(rAOGEN)123 have significantly elevated blood pressure compared with normal mice. Male transgenics develop higher pressure ( $159 \pm 8$  mmHg) than female animals ( $131 \pm 5$  mmHg) as compared with  $107 \pm 3$  mmHg for both male and female negative litter-mates. Animals of TGM(rAOGEN)92 had normal blood pressure.

#### **The transgene is expressed in different tissues**

The expression of the transgene as well as of the endogenous mouse AOGEN gene was analysed by RNase protection assay. This method discriminates between mouse and rat mRNAs. The RNA preparations were simultaneously hybridized to mouse and rat AOGEN antisense RNA. Initial experiments demonstrated the species specificity of the cRNA probes. No specific signals were obtained when the mouse probe was hybridized to rat RNA (Figure 5, lane rL) or vice versa (Figure 5, right panel).

The expression of the endogenous gene and the transgene was analysed in liver, brain, kidney, heart and testis of animals of both transgenic lines and of negative litter-mates (Figure 5). In TGM(rAOGEN)123 we detected rat AOGEN mRNA in all of the above organs. Expression was highest in liver and brain and detectable in kidney, heart and testis [Figure 5, panel TGM(rAOGEN)123]. When compared with the endogenous gene, transgene expression seemed to be higher in the brain and lower in the other organs with the exception of the liver [Figure 5, compare rAOGEN and mAOGEN signals in TGM(rAOGEN)123 panel].

The expression pattern of the transgene in animals of TGM(rAOGEN)92 was aberrant. Messenger RNA levels in the liver are at the limit of detection, when we use 10  $\mu$ g



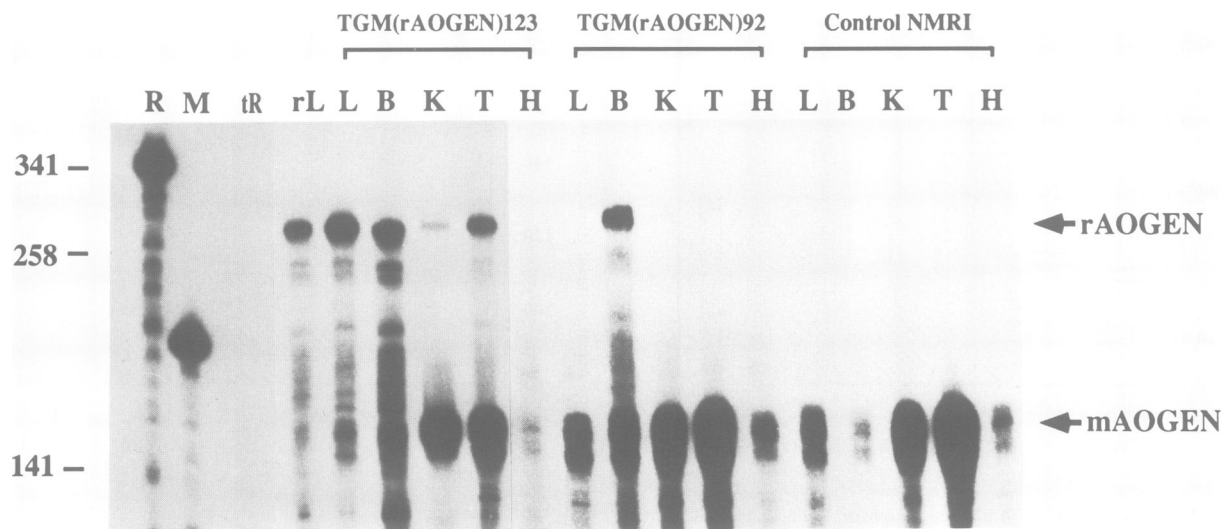
**Fig. 4.** Mean arterial blood pressure in transgenic mice. Control animals were either negative litter-mates or age-matched normal NMRI mice. Columns represent the mean  $\pm$  SEM of values obtained from five or more animals. For abbreviations see legend to Figure 2. The cross (+) indicates a significant difference between male and female animals of line 123.

of total liver RNA for hybridization. However, in the brain we detected considerable amounts of the mRNA, and weak signals were present in kidney, heart and testis [Figure 5, panel TGM(rAOGEN)92].

The RNase protection assay thus demonstrated that the transgene is expressed in a number of different organs. In order to study whether the expression occurs in a regional and cell type specific manner in the brain, we performed *in situ* hybridization on coronal sections of brain tissue of transgene positive animals of lines 123 and 92 as well as on negative control animals. In line 123, the transgene was expressed in specific areas of the brain (Figure 6A and B). The overall pattern was similar to the expression of the endogenous AOGEN gene in the rat (Bunnemann *et al.*, 1990). We detected high mRNA levels in the preoptic area (not shown) and in certain nuclei of the hypothalamus, e.g. in the paraventricular hypothalamic nucleus (Figure 6A, PHN), the suprachiasmatic nucleus (Figure 6A, SC), the supraoptic nucleus (Figure 6A, SON) and the arcuate nucleus (not shown). An expression pattern identical to the rat was found also in the locus ceruleus of the pons (not shown), in the Purkinje cell layer of the cerebellar cortex (Figure 6B, PL) and in the nucleus of the solitary tract (Figure 6B, NTS) and the inferior olive (Figure 6B, IO) of the medulla oblongata. There were, however, some differences as compared with the rat. The expression in some nuclei of the thalamus was higher in line 123 animals than in the rat (Figure 6A, paraventricular thalamic nucleus, PTN). The substantia nigra also showed high mRNA expression in transgenic animals whereas in the rat this area contains only moderate signals (not shown).

The expression pattern in the brain of animals of line 92 was very much different and comparable with the rat only in pons and medulla (Figure 6D). In the other parts of the brain, the very weak expression was restricted to the suprachiasmatic nucleus (Figure 6C, SC) and the rostral thalamus (not shown).

All signals observed were specific for rat AOGEN, since no hybridization was detected with the rat AOGEN probe in transgene negative mice or with a sense RNA probe in positive animals (data not shown).



**Fig. 5.** Expression of transgene and endogenous AOGEN gene in various organs of transgenic mice and normal NMRI controls. Total RNA of liver (L, 10  $\mu$ g), brain (B, 20  $\mu$ g), kidney (K, 100  $\mu$ g), testis (T, 100  $\mu$ g) and heart (H, 80  $\mu$ g) of animals of both transgenic lines or of negative control mice were hybridized overnight to a 200 bp mouse (M) and a 350 bp rat (R) AOGEN specific cRNA probe. Protected fragments of 150 bp (mAOGEN) and 290 bp (rAOGEN) are indicative for the presence of rat and mouse AOGEN mRNA, respectively. Total rat liver RNA (10  $\mu$ g, lane rL) and tRNA (80  $\mu$ g, lane tR) hybridized to both probes served as controls for specificity of the signals. The marker was *Sau*3A digested end labelled pUC19 DNA, relevant sizes are indicated on the left. Two different exposures of the same gel were used to visualize weak signals.

## Discussion

The generation of transgenic animals for the investigation of human genetic diseases has gained increasing interest in recent years (Connelly *et al.*, 1989; Hanahan, 1989). Transgenic animals have found entry also in cardiovascular and hypertension research (Mullins *et al.*, 1989, 1990; Ohkubo *et al.*, 1990; Steinhilper *et al.*, 1990; Field, 1991). The hypertensive transgenic rats TGR(mREN2)27, carrying the mouse REN 2 gene (Mullins *et al.*, 1990), were the first monogenic hypertension model and provide direct evidence that not only the classical circulating RAS but also local paracrine tissue RAS can lead to the development of hypertension.

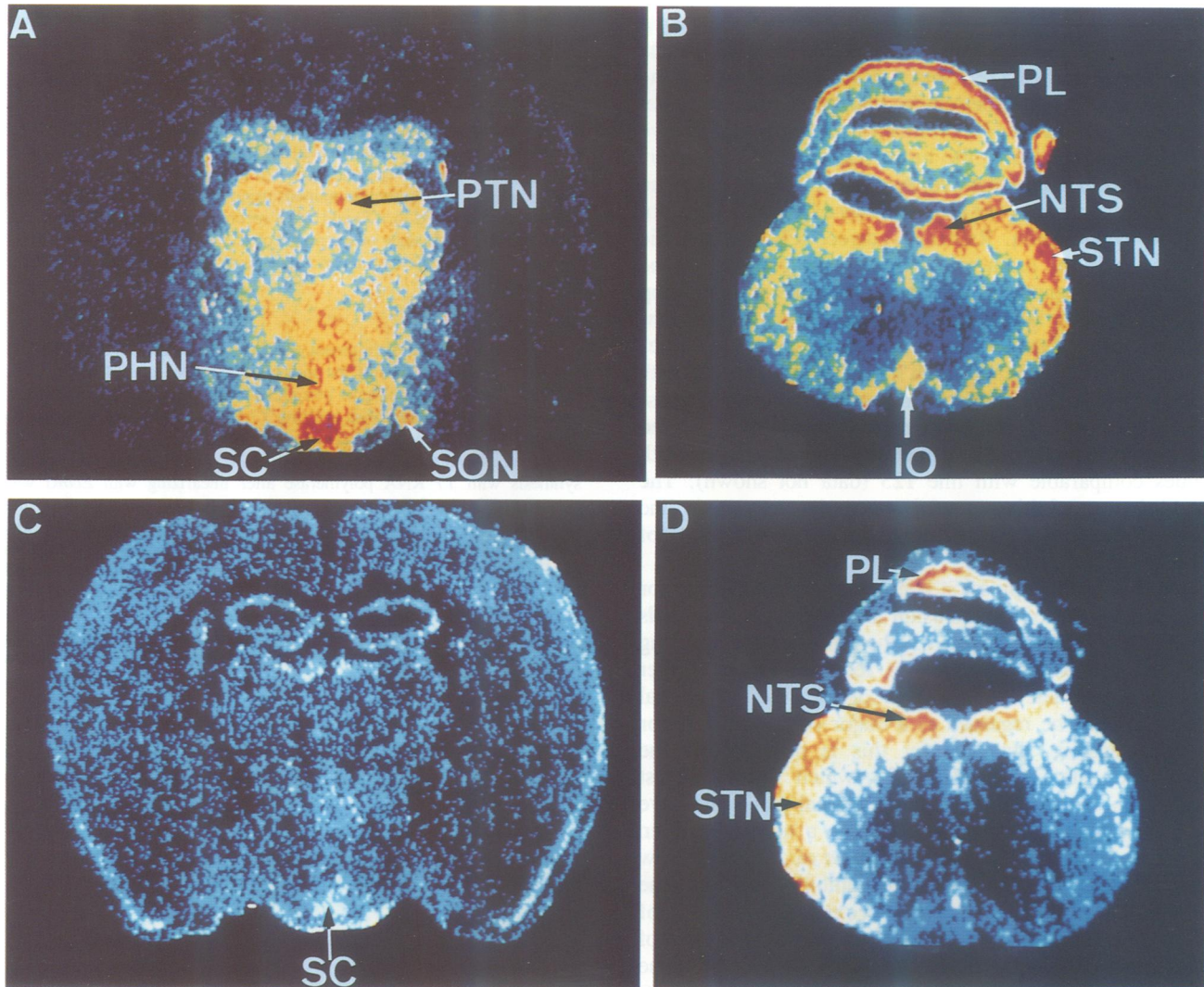
Our rationale for introducing the rat AOGEN gene expressed from its own promoter into the genome of the mouse was two-fold. First, mouse renin is capable of cleaving the rat AOGEN (Oliver and Gross, 1966). With an additional AOGEN gene, we would increase the circulating AOGEN concentration in the mouse, which was reported to be relatively low (Poulsen and Jacobsen, 1986). Since plasma substrate concentrations are the rate limiting factor for ANG II formation (Reid *et al.*, 1978), these animals could provide a model to study RAS kinetics *in vivo*. Second, using the natural promoter, we wanted to assure a correct tissue specific expression of the transgene, thereby providing a basis for a detailed analysis of cell specific gene expression. Studies with the mouse AOGEN gene (Clouston *et al.*, 1989) and the closely related  $\alpha$ 1-antitrypsin gene (Shen *et al.*, 1989) indicated that sequences sufficient for a correct expression of the AOGEN gene in transgenic animals reside within 1–2 kb upstream of the start of transcription. Such sequences should therefore be included in the 1.6 kb of 5' flanking sequences present in our transgene. In addition, we (Feldmer *et al.*, 1991) and others (Ron *et al.*, 1990a,b) have shown that promoter elements necessary for glucocorticoid, estrogen and cytokine induc-

tion are located within the first 700 bp of the start of transcription.

In TGM(rAOGEN)123 the transgene is expressed in liver and brain as well as in other tissues. In testis and kidney there seems to be a dissociation between the expression of the rat and the endogenous mouse AOGEN genes, the latter being predominantly expressed. In testis, this might reflect species specific differences in the level of expression, since in Sprague–Dawley rats, the mRNA concentration in testis is very low compared with other organs (Hellmann *et al.*, 1988). This is in contrast to the situation in some mouse strains (Clouston *et al.*, 1989). Differences between promoter elements mediating testis specific expression of the rat and the mouse genes might be responsible for this phenomenon. Whether the low expression of the transgene in the kidney is due to the lack of kidney specific *cis*-acting elements on the transgene promoter or whether it is caused by other regulatory mechanisms remains to be investigated. The lack of tissue specific expression in TGM(rAOGEN)92 is likely to be due to integration artifacts (Palmiter and Brinster, 1986). We can exclude a disruption of the transgene, especially loss of promoter sequences, from Southern analyses using promoter specific probes (data not shown). Position-dependent expression has also been reported for mouse AOGEN minigene constructs in transgenic mice (Clouston *et al.*, 1989) and might be attributed to transcriptional interference from nearby promoters (Proudfoot, 1986).

The data reported here strongly support the hypothesis that overexpression of AOGEN in a correct tissue specific manner can lead to hypertension. Transgene expression in the liver of TGM(rAOGEN)123 resulted in increased total plasma AOGEN and ANG II concentrations. The high rat AOGEN mRNA content of the brain was localized by *in situ* hybridization to areas and nuclei which are similar to the endogenous mRNA distribution in the rat (Bunnemann *et al.*, 1990). Some of these nuclei are important centres for cardiovascular control and all components of the RAS,





**Fig. 6.** Localization of rat AOGEN mRNA in the brain of transgenic mice. Serial coronal sections ( $7\ \mu\text{m}$ ) of brains of mice of line 123 (A and B) and line 92 (C and D) were hybridized to a rat AOGEN specific cRNA. Original autoradiographic signals were converted with an image analyser which allows for a semi-quantitative analysis. Signal intensities increase from light yellow to red, the latter indicating areas of highest mRNA concentrations. Sections of the rostral diencephalon (A and C) and the medulla oblongata (B and D) are shown to demonstrate the different expression patterns between the lines and to indicate important areas of expression: paraventricular hypothalamic nucleus (PHN), suprachiasmatic nucleus (SC), supraoptic nucleus (SON), paraventricular thalamic nucleus (PTN), Purkinje cell layer of the cerebellar cortex (PL), inferior olive (IO), nucleus of the solitary tract (NTS) and nucleus of the spinal tract of the trigeminal nerve (STN).

including AOGEN (Imboden *et al.*, 1987), ANG II immunoreactivity (Lind *et al.*, 1985) and ANG II receptors (Mendelsohn *et al.*, 1984) have been identified within these nuclei. Animals of TGM(rAOGEN)123 are hypertensive. In contrast, TGM(rAOGEN)92, which, apart from elevated plasma ANG II concentrations in males, did not show any of the characteristic parameters found in TGM(rAOGEN)123, were normotensive. The source of ANG II in line 92 males is unclear at present, since the transgene is only very weakly expressed in the liver and plasma AOGEN concentrations are similar to negative control mice. Plasma ANG II acts directly on vascular smooth muscle cells to increase vascular tone and, in addition, leads to water retention in the kidney by stimulation of aldosterone release from the adrenal gland. Both of these actions increase the blood pressure. Similarly, an overexpression of AOGEN in the brain is likely to result in increased central ANG II concentrations. This leads to an increase in sympathetic nervous system activity via stimulation of noradrenalin

release. In addition, modulating regulatory mechanisms in the periphery mediated by central release of humoral factors might be changed by high ANG II concentrations in the brain. Thus, an increased central ANG II concentration can also lead to hypertension. The exact mechanisms underlying a high ANG II mediated hypertension are not known, but the transgenic mice of TGM(rAOGEN)123 provide the opportunity to study these mechanisms and to gain information about the kinetics of the RAS *in vivo* under conditions of pharmacological intervention or further stimulation of the system. These studies will also help to elucidate the contribution of local tissue RAS to the development of hypertension. The circulating RAS is not the sole determinant of hypertension as suggested by the males of line 92 which are normotensive despite plasma ANG II levels similar to those of line 123 animals. Indirect evidence for the involvement of local tissue RAS is also provided by experiments of Ohkubo *et al.* (1990), who have generated transgenic mice with a rat AOGEN gene fused to the mouse

metallothionein I (mMT-I) promoter. Although the fusion gene was highly expressed in the liver and the total plasma concentrations of AOPEN and ANG II were similar to the values found in our hypertensive line, these mice were not hypertensive. The mMT-I promoter is predominantly active in the liver but also in the brain (Durham and Palmiter, 1981). This indicates that overexpression of AOPEN in the liver accompanied by an ectopic brain expression does not lead to hypertension. Rather, a correct cell type specific expression in the brain seems to be a prerequisite for the hypertensive phenotype. Only those transgenic mice develop this phenotype, which show correct brain expression. Preliminary data with a third transgenic line, TGM(rAOPEN)102, show that it is similar to line 123 with respect to plasma parameters and tissue distribution of transgene expression. Preliminary measurements on a limited number of animals revealed an elevated blood pressure with values comparable with line 123 (data not shown). The establishment of this second hypertensive transgenic line strongly supports the conclusions drawn from the data of TGM(rAOPEN)123.

Whether brain expression is necessary and sufficient for the development of hypertension in the transgenic animals of TGM(rAOPEN)123 or whether increased circulating ANG II concentrations are also required awaits further investigations. The RAS in other organs like the adrenal gland can also be regarded as a major contributing factor in hypertension. Evidence for this is provided by the transgenic rats TGR(mREN2)27 which show highest expression of the transgene in the adrenal gland and have extremely high blood pressure despite a normal or even suppressed plasma RAS (Mullins *et al.*, 1990). New methodologies like tissue specific inhibition of transgene expression in the renin overexpressing rats of TGR(mREN2)27 and the AOPEN overexpressing mice of TGM(rAOPEN)123 will help to clarify the role of tissue RAS versus plasma RAS in the development of hypertension.

## Materials and methods

### Generation of transgenic mice

The plasmid pRag15 U, containing the 14.2 kb *EcoRI*–*HindIII* fragment from the original 14.5 kb genomic rat AOPEN clone (Tanaka *et al.*, 1984) inserted into pUC19, was used to generate transgenic mice. This 14.2 kb fragment was purified from vector sequences on a 0.8% agarose gel, precipitated, redissolved in 10 mM Tris–HCl (pH 7.4)/0.2 mM EDTA and filtered through 0.22 µm sterile filter tips to remove impurities. The DNA (1 µg/ml) was injected into fertilized oocytes of NMRI mice.

### Southern blotting

Ten µg of DNA obtained from tail biopsies of 3–4 week old mice were digested with *PvuII*, separated on a 0.8% agarose gel and processed for Southern hybridization by vacuum blotting onto Nylon Ny 13N membranes (Schleicher and Schüll). The probe used was a 712 bp *BamHI* fragment of the rat AOPEN cDNA clone pRag16 (Ohkubo *et al.*, 1983). It was radiolabelled with [ $\alpha$ -<sup>32</sup>P]dCTP using the random prime method (Feinberg and Vogelstein, 1983). Hybridization was carried out overnight at 65°C and the membrane was exposed to X-ray film with an intensifying screen at –80°C.

### Parameters of the RAS

Determination of rat AOPEN concentration in plasma was done by ELISA as described (U. Hilgenfeldt, S. Schwind, T. Muley and I. Rubin, submitted), using the monoclonal antibody A1-F6 directed against human AOPEN (Rubin *et al.*, 1988). Total plasma AOPEN concentration was determined by indirect RIA via the generation of ANG I as described by Schelling *et al.* (1980) with slight modifications. Plasma was incubated for a period of 3 h with a 1:10 diluted kidney extract of NMRI mice which served as a source

of renin. Three hours were found to be sufficient to cleave quantitatively both rat and mouse AOPEN. Plasma ANG II concentration was measured by RIA essentially as described (Hermann *et al.*, 1988).

### Blood pressure measurements

Blood pressure measurements were performed by inserting a PE 10 catheter into the femoral artery of the animals under pentobarbital anesthesia (Jacob *et al.*, 1991). After the operation (15–25 h) mean arterial blood pressure was recorded on the conscious animals using a Stratham P23 D3 pressure transducer connected to a Gould 2400 recorder. Pressure and heart rate were recorded continuously over a period of 15 min. Animals were sacrificed thereafter and organs and blood were taken and processed for further experiments.

### RNA analysis

A 290 bp *PvuII*–*BamHI* fragment of pRag16 was cloned into the *BamHI* and *SmaI* restriction sites of the transcription vector pGEM 4 (Promega Biotech). The resulting plasmid pRag0.3G4 was linearized with *EcoRI* and then used for cRNA synthesis utilizing T7 RNA polymerase according to the method of Melton *et al.* (1984).

The mouse AOPEN probe pMag0.15G4z which allowed for cRNA synthesis with T7 RNA polymerase after linearizing with *EcoRI* was constructed in the following way. A 6.3 kb *BamHI* fragment from the genomic mouse AOPEN clone mAO (R. Metzger, unpublished) was subcloned into Bluescript SK II (Stratagene) and subsequently digested with *EcoRI* and *BglIII*. The resulting 158 bp fragment was isolated and cloned into the *EcoRI* and *BamHI* sites of pGEM4z (Promega Biotech) to yield the plasmid pMag0.15G4z.

RNAse protection assays were performed essentially as described by Mullins *et al.* (1990). Briefly, total RNA was extracted from different tissues using the LiCl/urea method (Auffray and Rougeon, 1980). Hybridization was carried out overnight with  $2 \times 10^5$  c.p.m. each of freshly labelled antisense probes at 65°C. RNAse resistant hybrids were separated on a 5% sequencing gel, which was then exposed to X-ray film at –80°C.

### In situ hybridization

*In situ* hybridization on mouse brain was performed as previously described (Bunnemann *et al.*, 1990), using the plasmid pRag0.3G4 and [ $\alpha$ -<sup>35</sup>S]UTP to synthesize the cRNA probe. Radioactive signals were converted with an image analyser (SAS Brovision, Milan, Italy).

### Statistical analyses

Statistical analysis of data was performed with Student's *t* test or Anova test. Differences were considered significant at  $P < 0.05$  indicated by an asterisk (\*). Two asterisks indicate a value of  $P < 0.01$ .

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